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Production of 22:2$^{\Delta 5,\Delta 13}$ and 20:1$^{\Delta 5}$ in *Brassica carinata* and soybean breeding lines via introduction of *Limnanthes* genes

Ashok Jadhav[1], Elizabeth-France Marillia[1], Vivijan Babic[1], E. Michael Giblin[1], Edgar B. Cahoon[2], Anthony J. Kinney[3], Elzbieta Mietkiewska[1], Jennifer M. Brost[1] and David C. Taylor[1,*]

*[1]National Research Council of Canada, Plant Biotechnology Institute, 110 Gymnasium Place, Saskatoon, Saskatchewan, Canada S7N OW9; [2]USDA-ARS, Plant Genetics Research Unit, Donald Danforth Plant Science Center, 975 N. Warson Road, St. Louis, Missouri 63132; [3]Dupont Experimental Station, P.O. Box 80402, Wilmington, Delaware 19880-0402; *Author for correspondence (e-mail: David.Taylor@nrc-cnrc.gc.ca; phone: +1-306-975-5268; fax: +1-306-975-4839)*

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Abstract

Seed oils of meadowfoam (*Limnanthes douglasii*, *L. alba*) contain very long-chain fatty acids of strategic importance for a number of industrial applications. These include the monoene 20:1$^{\Delta 5}$ and the diene 22:2$^{\Delta 5,\Delta 13}$. Engineering of meadowfoam-type oils in other oilseed crops is desirable for the production of these fatty acids as industrial feedstocks. Accordingly, we have targeted *Brassica carinata* and soybean (*Glycine max*) to trangenically engineer the biosynthesis of these unusual fatty acids. An *L. douglasii* seed-specific cDNA (designated *Lim Des*5) encoding a homolog of acyl-coenzyme A desaturases found in animals, fungi and cyanobacteria was expressed in *B. carinata*, which resulted in the accumulation of up to 10% 22:2$^{\Delta 5,\Delta 13}$ in the seed oil. In soybean, co-expression of *Lim Des5* with a cDNA (*Lim FAE1*) encoding an FAEl (elongase complex condensing enzyme) homolog from *L. douglasii* resulted in the accumulation of 20:1$^{\Delta 5}$ to approximately 10% of the total fatty acids of seeds. The content of C_{20} and C_{22} fatty acids was also increased from $<0.5\%$ in non-transformed soybean seeds to $>25\%$ in seeds co-expressing the *Lim. douglasii Des5* and *FAE1* cDNAs. In contrast, expression of the *Lim Des5* in *Arabidopsis* did not produce the expected 20:2$^{\Delta 5,\Delta 11}$ in the seed oil. Cumulatively, these results demonstrate the utility of soybean and *B. carinata* for the production of vegetable oils containing novel C_{20} and C_{22} fatty acids, and confirm that the preferred substrates of the *Lim Des5* are 20:0 and 22:1$^{\Delta 13}$, respectively.

Abbreviations: Lim Des5 – Limnanthes Acyl-CoA Δ 5 desaturase; *Lim FAEl – Limnanthes* elongase complex condensing enzyme; CoA – Coenzyme A; DEA – Diethylamide; DW – dry weight; ER – endoplasmic reticulum; FAME – fatty acid methyl ester; FFA – free fatty acid; PC – phosphatidylcholine; PE – phosphatidylethanolamine; TAG – triacylglycerol; TLC – thin layer chromatography; VLCFA – very long-chain fatty acid

Introduction

We are investigating possible sources of new genes to engineer unusual fatty acids in *Brassicaceae* and soybean. One target is the capacity to desaturate very long-chain fatty acids (VLCFAs) at the $\Delta 5$ position. A number of plants produce seed oils enriched in unusual fatty acids with a $\Delta 5$ functionality, including species of meadowfoam: *Limnanthes douglasii* and *L. alba*. *Limnanthes* seed oils are enriched in $\Delta 5$-eicosenoic acid ($20{:}1^{\Delta 5}$) and, to a much lesser extent, an unusual diene, $\Delta 5$, $\Delta 13$-docosadienoic acid ($22{:}2^{\Delta 5,\Delta 13}$) (Phillips et al. 1971). Because of their unique double bond positioning, both of these fatty acids are of strategic interest as industrial feedstocks. Its oxidative stability and high content of VLFCAs impart to the seed oil of *Limnanthes* species a number of properties that are desired by the cosmetic, surfactant, and lubricant industries (Hirsinger 1989; Burg and Kleiman 1991; Isbell et al. 1999). The $20{:}1^{\Delta 5}$ component of this oil can also serve as a chemical precursor of compounds such as estolides and δ-lactones that can be used for a wide range of industrial applications, including lubricants and plasticizers (Erhan et al. 1993; Isbell and Plattner 1997). The relatively high price of meadowfoam oil, however, limits its commercial use to primarily cosmetic applications, and as a result, this plant is currently grown only as a niche crop in the Pacific Northwest of the United States (Hirsinger 1989).

The proposed biosynthetic pathway for $20{:}1^{\Delta 5}$ in *Limnanthes* species involves three steps (Pollard and Stumpf 1980; Moreau et al. 1981): a flux of palmitic acid (16:0) from the plastid to the ER, followed by microsomal elongation of 16:0 to eicosanoic acid, and the $\Delta 5$ desaturation of 20:0 to yield $20{:}1^{\Delta 5}$.

We have recently confirmed this pathway by identification of cDNAs for a $\Delta 5$ desaturase (*Lim Des 5*) and a divergent FAE1 (elongase complex condensing) homolog (*Lim FAE1*) from *L. douglasii* (Cahoon et al. 2000). These cDNAs were co-expressed in soybean somatic embryos to produce $20{:}1^{\Delta 5}$ (Cahoon et al. 2000).

The small but significant proportion of a unique diene, $22{:}2^{\Delta 5,\ \Delta 13}$ in meadowfoam oil (10–15%; Miller et al. 1964; Phillips et al. 1971) is also of industrial interest. This diene possesses widely spaced, non-conjugated or methylene-interrupted double bonds making it quite stable and not as prone to oxidation. There are niche market applications that have been identified for its use as a feedstock for generating estolides, which can be used to synthesize hydroxy fatty acid feedstocks, and to produce dimer acids, esters and amides for use as lubricants, and slip-promoting anti-block agents in plastic film manufacturing (Burg and Kleiman 1991; Erhan et al. 1993). The proposed pathway for $22{:}2^{\Delta 5,\Delta 13}$ biosynthesis is thought to involve $\Delta 5$ desaturation of erucic acid, $22{:}1^{\Delta 13}$.

In the current study, *B carinata* and soybean were tested as potential 'transgenic vehicle' crops, for engineering the production of $22{:}2^{\Delta 5,\Delta 13}$ and $20{:}1^{\Delta 5}$, respectively. An *L. douglasii* seed-specific cDNA (*Lim Des5*) encoding a homolog of acyl-coenzymeA (CoA) desaturases found in animals, fungi and cyanobacteria, was heterologously expressed in *B. carinata*. In addition, this cDNA as well as one (*Lim FAE1*) encoding an (elongase complex condensing enzyme) homolog from *L. douglasii* were co-expressed in somatic soybean embryos.

Materials and methods

General molecular biology techniques and analyses of transgenic plants

Unless otherwise stated, all molecular biological techniques (plasmid preparation, PCR, Southern and northern analyses, etc.) were carried out by methods prescribed by Sambrook et al. (1989) or Ausubel et al. (1995).

Transgenic expression of the Limnanthes Des5 in Brassica carinata

The plant transformation vector pSE 129A, already prepared from pRD400 plasmid (Datla et al. 1992), was obtained by introducing a *Hind*III/*Xba*I fragment containing the *B. napus* napin promoter and a *Kpn*I/*Eco*RI fragment containing the *Agrobacterium nos* terminator. The 1.0 kb open-reading frame of the *Lim Des5* (*Limnanthes* Acyl-CoA $\Delta 5$ desaturase, GenBank Accession no AF247133) was amplified by PCR designed to contain *Xba*I and *Kpn*I restriction sites and was ligated into *Xba*I/ *Kpn*I-digested pSE129A in the sense orientation. The sense construct *Lim des5*/pSE was then

introduced by electroporation into *Agrobacterium tumefaciens* strain GV3101 bearing the helper plasmid pMP90 (Koncz and Schell 1986) for plant transformation.

Brassica carinata breeding line # C90-1163, kindly provided by Dr. G. Rakow, Agriculture and Agri-Food Canada, Saskatoon, was the selected line for transgenic studies. This line contains about 42% erucic acid; seed oil content is about 30% DW. Five-day-old cotyledons were transformed according to the method of Babic et al. (1998). All experimental control and transgenic *B. carinata* lines were grown simultaneously (Kristjanson Biotechnology Complex greenhouses, Saskatoon) under natural light conditions supplemented with high-pressure sodium lamps with a 16 h photoperiod (16 h of light and 8 h of darkness) at 22 °C and a relative humidity of 25 to 30%.

Transgenic co-expression of the Limnanthes Des5 and FAE1 in soybean seeds

The open-reading frames of the *Lim Des5* and *Lim FAEl* (Limnanthes *FAE*, GenBank Accession, no AF247134) cDNAs (Cahoon et al. 2000), were initially introduced as *Not*I restriction fragments into separate plant expression plasmids that contained strong seed-specific promoters. The *Lim Des5* cDNA was linked at its 5′ ends to the promoter for the soybean α' subunit of β-conglycinin gene (Doyle et al. 1986) and its 3′ end to the 3′ non-translated sequence of the phaseolin gene (Doyle et al. 1986). The *Lim FAE1* cDNA was linked at its 5′ end to the promoter for the soybean Kunitz trypsin inhibitor-3 (KTI-3) gene (Jofuku and Goldberg 1989) and its 3′ end to the 3′ non-translated sequence of the KTI-3 gene (Jofuku and Goldberg 1989). The resulting *Lim Des5* and *Lim FAE1* expression cassettes were then assembled in tandem into a single plasmid, designated pKR21. The two expression cassettes were linked at the 5′ ends of their respective promoters, which resulted in an opposite (or 'head to head') orientation of the *Des5* and *FAEl* cassettes in pKR21. The tandem expression cassettes were flanked by *Asc*I restriction sites.

For transformation experiments, pKR21 was digested with *Asc*I, and the fragment containing the tandem expression cassettes was purified by agarose gel electrophoresis. The purified fragment from pKR21 was used for soybean transformation along with a second DNA fragment that contained a hygromycin phosphotransferase gene (Gritz and Davies 1983) under control of the cauliflower mosaic virus 35S promoter. The two fragments were introduced into somatic embryos of soybean [*Glycine max* (L.) Merr. cv Jack] by biolistic transformation as described (Finer and McMullen 1991; Jung and Kinney 2001). The molar ratio of the pKR21-derived fragment to the hygromycin selection fragment used in the transformation was approximately 10:1. Following transformation, the soybean somatic embryos were maintained and propagated as described (Jung and Kinney 2001). Hygromycin-resistant embryos obtained from the transformation were screened for the production of $20{:}1^{\Delta 5}$ by gas chromatographic analysis of fatty acid methyl esters (FAMEs) prepared from these tissues (Cahoon et al. 2003). Trait-positive lines were regenerated as described (Jung and Kinney 2001). The presence of the *Lim Des5* and *Lim FAE1* transgenes and their copy number were determined by Southern blot analysis of genomic DNA prepared from leaves of regenerated plants. Seeds for lipid analyses were obtained from plants grown at 27 °C/23 °C (day/night) with 50% relative humidity and maintained at a day length of 16 h.

Lipid analyses

Ten T_1 plants were propagated from each primary transgenic event. Mature T_2 seeds from *B. carinata Lim Des5* plants and from non-transformed wild-type control plants were sampled and subjected to analyses of total fatty acid content and composition. Seed samples were ground using a polytron in chloroform-isopropanol (2:1 v/v) containing tripentadecanoin as an internal standard. All other conditions for the extraction and gas chromatography analyses of seed lipids from greenhouse-grown plants for total fatty acid content and fatty acid composition (expressed as % (wt/wt) of total fatty acids) were performed as described previously (Katavic et al. 1995; Zou et al. 1997; Cahoon et al. 2000; Marillia et al. 2002).

In the case of *B. carinata Lim Des5* transgenics, a line containing about 10% of the putative $\Delta 5$ product $22{:}2^{\Delta 5,\Delta 13}$ was selected and the product

authenticated by GC-MS as described previously (Marillia et al. 2002). Briefly, following saponification of a portion of seed oil, diethylamide (DEA) derivatives were prepared from free fatty acids (FFAs) using the method described by Nilsson and Liljenberg (1991), for GC-MS analyses. The DEAs were evaporated to dryness under nitrogen, dissolved in 200 μl acetonitrile and filtered using a Spin-X filter # 8169 (Corning Incorporated, Corning, NY 14831). The DEAs were fractionated by HPLC using two 125×4.6 min reverse-phase C_{18} Partisphere columns (Chromatographic Specialties Inc.) in tandem. Fractions containing the $16{:}1^{\Delta 5}$, $18{:}1^{\Delta 5}$, $20{:}1^{\Delta 5}$ and $22{:}2^{\Delta 5,\Delta 13}$ DEAs were collected every minute (HPLC flow rate of 1 ml/min) beginning 3 min before and continuing for 3 min after the retention time of the authentic standard DEA derivatives. Fractions were evaporated under nitrogen, resuspended in 50μl hexane and a small aliquot checked for the presence of the expected $\Delta 5$ fatty acid DEA derivatives by injection on the GC prior to establishing the $\Delta 5$ regioselectivity of the products by GC-MS as described by Nillson and Liljenburg (1991).

In addition, seed samples from the high $22{:}2^{\Delta 5,\Delta 13}$-producing lines were analyzed with respect to triacylglycerol (TAG) and phosphatidylcholine (PC) fatty acid composition as described previously (Taylor et al. 1992).

The fatty acid composition of mature soybean seeds from non-transformed plants or from plants transformed with the *Lim Des5* and *Lim FAE1* cDNAs (T_5 generation) was determined by analysis of FAMEs derived from chipped portions of single seeds as described (Cahoon et al. 2003). Seed chips were incubated directly in trimethylsulfonium hydroxide/methanol for transesterification of lipids using previously described methodology (Cahoon et al. 2003). The resulting FAMEs were analyzed by GC using an Agilent 6890 gas chromatograph fitted with a DB23 column (30-m-$\times$ 0.25-mm, inner diameter; Agilent). The oven temperature was programmed from 180 °C (2-min hold) to 225 °C at 5 °C/min. Eluted FAMEs were detected and quantified by flame ionization. Double bond positions of C_{20} and C_{22} unsaturated fatty acids were determined by GC-MS analysis of DEA derivatives of FFAs from transgenic seeds as described (Nilsson and Liljenberg 1991). The identities of novel fatty acids were also determined by comparison of GC retention times of FAMEs from transgenic soybean seeds with those of C_{20} and C_{22} FAMEs of known identity from *L. douglasii* seeds.

For analysis of the fatty acid content of lipid classes, total lipids were extracted from single soybean seeds that had been homogenized with a mortar and pestle. Lipid extraction was conducted according to the procedure described by Bligh and Dyer (1959). Neutral lipids were resolved on silica gel 60 thin layer chromatography (TLC) plates (Merck) with a solvent system consisting of heptane:ethyl ether:acetic acid (60:40:1 v/v/v). The TAG fraction was identified by mobility relative to a corresponding standard and detected by light staining with iodine vapor. The TAG band was scraped from the plate into a 13 mm $\times$ 100 screw cap test tube that contained 1-ml of 2.5% (v/v) sulfuric acid/methanol and 300-μl of toluene. The capped tube was incubated at 90 °C for 45 min. FAMEs were recovered by heptane extraction following the addition of 1-ml of 1 M sodium chloride to the cooled reaction. FAMEs were then analyzed by GC as described above. For fatty acid analyses of phospholipid classes, the total lipid extract was resolved by silica TLC with a solvent system consisting of chloroform:methanol:acetic acid (65:30:8 v/v/v). Bands recovered from TLC plates corresponding to PC and phosphatidlyethanolamine (PE), as determined by mobility relative to standards, were reacted in 1-ml of 2.5% (v/v) sulfuric acid at 90 °C for 30 min. FAMEs were subsequently recovered and analyzed by GC, as described for the characterization of TAG fatty acid composition.

Results

Production of $22{:}2^{\Delta 5,\Delta 13}$ in transgenic B. carinata

Cotyledons of *B.carinata* were transformed with the *Lim Des5* desaturase gene construct and putative transformants grown on selective media (kanamycin resistance). Following selection, 37 transgenic lines were identified. DNA was isolated from all transgenic lines and PCR analysis was performed to reconfirm the presence of the *Lim Des5* desaturase gene by using both gene and promoter-specific primers. Out of 37 transformants, 28 showed positive PCR results.

In Southern analyses, DNA was digested with *Eco*RI (which does not have restriction sites within the gene) and hybridization was carried out at 65 °C with the *Lim Des5* desaturase gene. The number of copies inserted in each line was scored. As expected, the control did not show any hybridization. On the basis of inserted copy numbers, *Lim Des5* desaturase T_1 transformants were grouped into three main categories: one copy (5 plants), two copies (7 plants) and three copies (13 plants). The inserted copy sizes ranged from 3 to 10 kb. Segregation analyses of the transformant progeny showed similar results to the Southern analysis.

GC analyses of FAMEs prepared from the T_1 seeds of transgenic plants of *B. carinata*, showed the presence of both $20{:}1^{\Delta 5}$ and $22{:}2^{\Delta 5,\Delta 13}$ with the diene being about 10-fold more prominent than the monoene (Table 1). These unique very long-chain mono- and di-unsaturated fatty acids were absent in seed oils from the non-transformed control plants. Most transgenics also contained small but detectable proportions (< 0.1%) of $20{:}2^{\Delta 5,\Delta 11}$. Ten plants from each T_1 line were propagated in the greenhouse. Developing seed of the T_2 generation were analyzed by northern blot for relative expression of the $\Delta 5$ desaturase. A strong positive correlation was observed between *Lim Des5* transcript intensity in the developing embryos (Figure 1) and the average 22:2 content in the mature progeny (T_2 seed lines; Figure 2). Segregating progeny from 31 T_2 lines accumulated, proportions of $22{:}2^{\Delta 5,\Delta 13}$ ranging from 1 to > 10% of the total fatty acid pool in the mature seed oil, chiefly at the cumulative expense of erucic acid ($22{:}1^{\Delta 13}$) and its precursors, oleic acid followed by eicosenoic acid ($20{:}1^{\Delta 11}$). Sandager and Stymne (2000) have suggested that in meadowfoam, there is a second *FAE1* (independent of that cloned by Cahoon et al. 2000) that is more specific for the elongation of $18{:}1^{\Delta 9}$ to eicosenoic and then erucic acid. Selected T_2 segregant seed lines displayed three ranges of $22{:}2^{\Delta 5,\Delta 13}$ accumulation: low (1 to 3%), medium (4 to 6%) and high (7 to 10.7%) as shown in Figure 3. Southern hybridization showed a strong correlation between the number of *Des5* copies inserted and the resulting production of $22{:}2^{\Delta 5,\ \Delta 13}$. Lines with the highest $22{:}2^{\Delta 5,\Delta 13}$ (7 to > 10%) contained three copies, those with medium proportions of $22{:}2^{\Delta 5,\Delta 13}$ (4 to 6%) contained two copies, and lines with relatively low production of $22{:}2^{\Delta 5,\Delta 13}$ (< 2%) had only one copy of the gene.

Table 1. Fatty acid composition of oils from transgenic *B. carinata* T_1 seed expressing the *Lim Des5* gene (+ *Des5*) and non-transformed *B. carinata* seed. The data obtained are the average ± SD of 12-seed samplings from eight plants for the transgenics and two plants in the case of controls.

Fatty acid	Non-transformed (n = 2)	+ *Des5* (n = 8)
	Weight % of total fatty acids	
16:0	3.1 ± 0.1	4.5 ± 0.4
18:0	0.7 ± 0.02	0.9 ± 0.1
$18{:}1^{\Delta 9}$	8.4 ± 0.2	7.3 ± 1.3
$18{:}2^{\Delta 9,\Delta 12}$	16.5 ± 0.6	20.0 ± 2.5
$18{:}3^{\Delta 9,\Delta 12,\Delta 15}$	15.2 ± 0.6	16.8 ± 1.8
20:0	0.6 ± 0.09	0.4 ± 0.1
$20{:}1^{\Delta 5}$	ND[a]	0.5 ± 0.1
$20{:}1^{\Delta 11}$	5.1 ± 0.1	5.9 ± 1.1
$20{:}2^{\Delta 11,\Delta 14}$	1.0 ± 07	1.1 ± 0.01
$20{:}2^{\Delta 5,\Delta 11}$	ND[a]	Tr[b]
22:0	0.8 ± 0.2	0.3 ± 0.1
$22{:}1^{\Delta 13}$	43.0 ± 0.7	35.0 ± 1.4
$22{:}2^{\Delta 13,\Delta 16}$	0.4 ± 0.05	0.2 ± 0.05
$22{:}2^{\Delta 5,\Delta 13}$	ND[a]	4.8 ± 0.5
Total C_{20} + C_{22}	50.9	48.2

[a]ND, Not detected. [b] Tr, trace; < 0.1%.

The regiospecificity of the *Lim Des5* desaturase was verified by GC-MS of the DEA derivative of $22{:}2^{\Delta 5,\Delta 13}$ isolated from transgenic lines 12–5, 14–8 and 16–9 which strongly expressed the desaturase gene. The results confirmed the regioselectivity of the transgene product. On a mass spectrum, typically, fragments of n and n + 1 carbons differing by 12 D reveal a double bond between carbons n + 1 and n + 2 (Nilsson and LiIjenberg 1991). Therefore, the fragments at 142 and 154 and at 252 and 264 shown in the mass spectrum of 22:2 from line 14–8, reveal double bonds at the Δ^{5-6} and Δ^{13-14} positions, respectively, in a 22-carbon di-unsaturated fatty acid DEA derivative ($M^+ = 391$) (Figure 4). The identical fragmentation pattern was observed in the mass spectrum of the authentic standard of the $22{:}2^{\Delta 5,\Delta 13}$ DEA derivative (data not shown).

The fatty acid composition of the TAG and PC classes were compared in *B. carinata* T_2 seeds of lines 14–8, 12–5 and 16–9 expressing the *Lim Des5* (Table 2). Results showed that while the proportions of $20{:}1^{\Delta 5}$ were about equal in both TAG and PC, the proportion of $22{:}2^{\Delta 5,\Delta 13}$ was almost 10-fold higher in TAG (10.7%) compared to PC (1.2%).

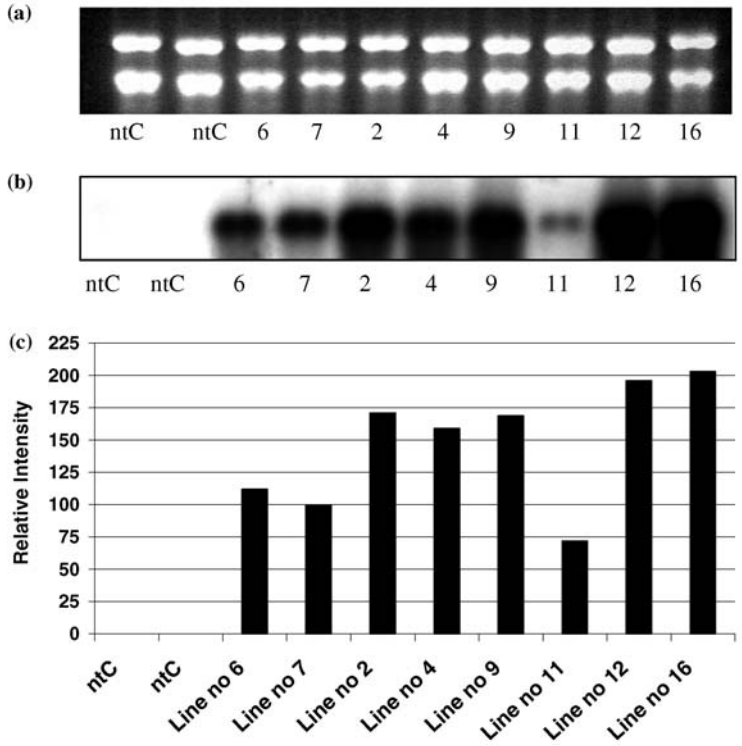

Figure 1. Northern expression of *Des 5 in B. carinata* pooled T_2 developing seeds. (a) Relative loading of total RNA as estimated by intensity of rRNA bands in seeds of non-transformed control plants (NtC) and seeds of transgenic line numbers 6, 7, 2, 4, 9, 11, 12, 16, as indicated. (b) *Lim Des 5* transcript signal in NtC and transgenic lines. (c) Relative intensity of *Lim Des 5* transcript in ntC and each of the transgenic lines (with signal for line number 7 set at 100%). The $22{:}2^{\Delta 5,\Delta 13}$ content in the seed oil of the transgenic lines are as follows: line number 6, $<2\%$; line number 7, $<2\%$; line number 2, 2–4%; line number 4, 2–4%; line number 9, 6%; Line number 11, $<1.5\%$; Line number 12, 9%; Line number 16, 10%.

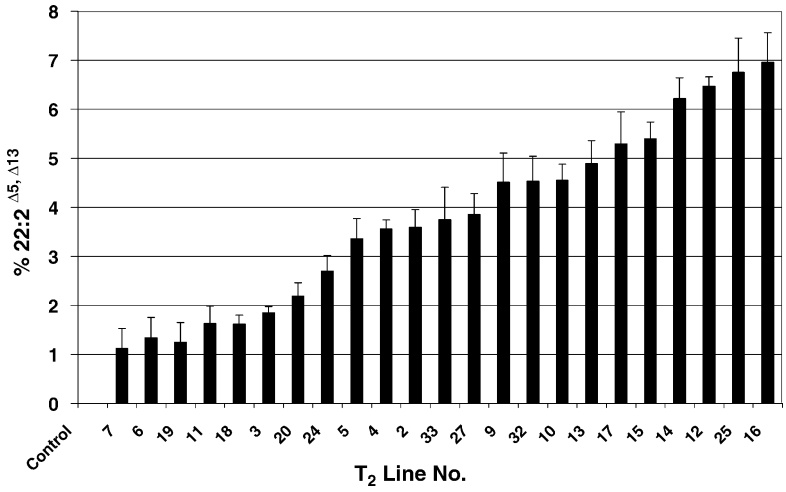

Figure 2. Average proportions of $22{:}2^{\Delta 5,\Delta 13}$ in T_2 seed oil of *B. carianta* transformed with the *Lim Des 5* gene. Ten T_1 plants were propagated from each primary transgenic event. 12-seed lots were sampled from each plant and analyzed as described. The data represent the 22:2 content ($\pm$SE) as a combined average for T_2 seed obtained from all 10 segregants of each line.

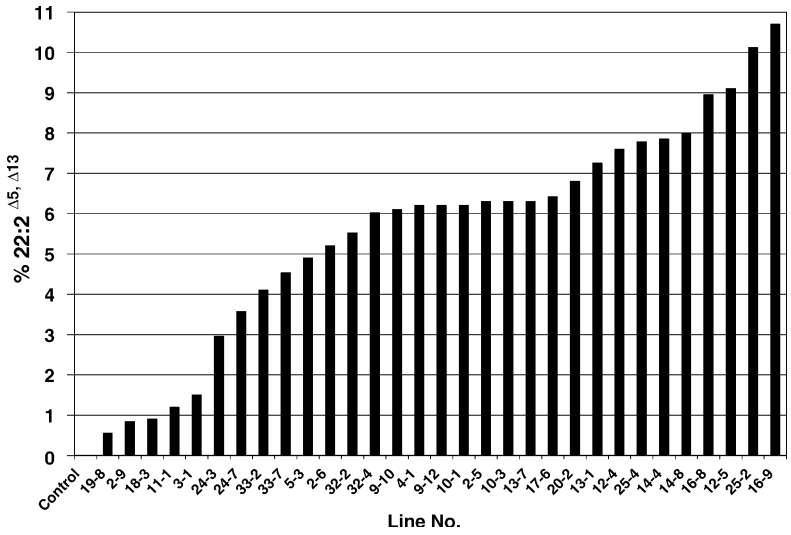

Figure 3. Proportions of $22{:}2^{\Delta 5,\Delta 13}$ in selected T_2 progeny of *B. carianta* transformed with the *Lim Des 5*. Values are the average of 22:2 found in seed oil from 12-seed lots sampled in triplicate from individual T_2 segregants.

Table 2. Fatty acid composition of triacylglycerol (TAG) and phosphatidylcholine (PC) in *B. carinata* T_2 seeds of lines 14–8, 12–5 and 16–9 expressing the *Lim Des5*. Measurements are expressed as weight % of total fatty acids of each lipid class ± SD.

Fatty acid	TAG (n = 3)	PC (n = 3)
	Weight % of total fatty acids	
16:0	3.2 ± 0.2	20.9 ± 0.1
18:0	1.2 ± 0.2	3.2 ± 0.4
$18{:}1^{\Delta 9}$	8.5 ± 0.2	5.3 ± 0.02
$18{:}2^{\Delta 9,\Delta 12}$	19.0 ± 0.2	45.6 ± 0.5
$18{:}3^{\Delta 9,\Delta 12,\Delta 15}$	10.3 ± 0.4	9.6 ± 0.2
20:0	0.6 ± 0.01	0.4 ± 0.02
$20{:}1^{\Delta 5}$	1.0 ± 0.02	1.0 ± 0.01
$20{:}1^{\Delta 11}$	5.7 ± 0.1	2.7 ± 0.2
$20{:}2^{\Delta 5,\Delta 13}$	Tr[a]	ND[b]
22:0	0.4 ± 0.01	Tr[a]
$22{:}1^{\Delta 13}$	32.9 ± 0.1	3.9 ± 0.1
$22{:}2^{\Delta 5,\Delta 13}$	10.7 ± 0.02	1.2 ± 0.01
$22{:}2^{\Delta 13,\Delta 16}$	1.2 ± 0.02	0.3 ± 0.01
24: $1^{\Delta 15}$	1.5 ± 0.01	0.4 ± 0.01
Other[c]	1.6	3.2
Total C_{20}, C_{22}, C^{d}_{24}	55.5	10.4

[a]Tr, trace; <0.1%; [b] ND, Not detected; [c] Includes $16{:}1^{09}$, $18{:}1^{\Delta 11}$ and 26:0; [d] Includes $20{:}1^{\Delta 13}$, 20:3 and 24:0.

Production of VLCFAs in soybean seeds

Fatty acid compositional analyses were conducted using soybean seeds from a line containing a single copy of the *Lim Des5* and *Lim FAE1* transgenes, as determined by Southern blot analysis (data not shown). Consistent with the expression of both genes, $20{:}1^{\Delta 5}$ was produced in seeds of the transgenic line but was absent from seeds of non-transformed plants. This fatty acid accumulated to approximately 10% of the total fatty acids (Table 3). Trace amounts of other Δ^5 unsaturated fatty acids were detected, including $22{:}1^{\Delta 5}$ and $22{:}2^{\Delta 5,\Delta 13}$. In addition, C_{20} and C_{22} fatty acids derived from oleic and linoleic acids were detected in seeds of the transgenic line, including primarily $20{:}1^{\Delta 11}$ and $20{:}2^{\Delta 11,\Delta 14}$. Overall, C_{20} and C_{22} fatty acids comprised >25% of the total fatty acids of soyabean seeds from seeds co-expressing the *Lim Des5* and *Lim FAE1* transgenes (Table 3). By contrast, these fatty acids comprised <0.5% of the fatty acids of seeds from non-transformed plants. Accumulation of the VLCFAs in seeds from the transgenic line occured primarily at the expense of 16:0 and 18:0. Amounts of these fatty acids decreased from 16% of the total fatty acids of seeds from non-transformed plants to 6% in seeds of transgenic plants.

The fatty acid composition of the lipid classes of seeds co-expressing the *Lim Des5* and *Lim FAE1* cDNAs was also examined (Table 4). Although $20{:}1^{\Delta 5}$ was present in the major phospholipid classes PC and PE, amounts of this fatty acid in PC and PE were at least three- to 5-fold lower than that detected in TAG. A similar difference in total

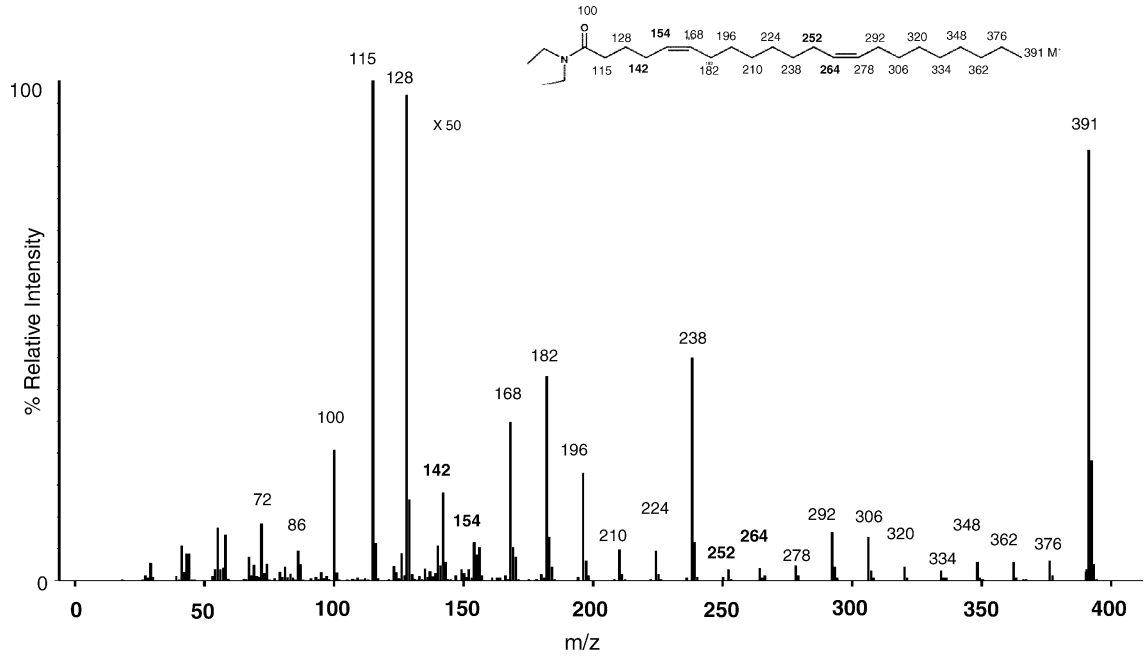

Figure 4. Mass spectrum of the diethylamide derivative of 22:2$^{\Delta 5,\Delta 13}$ in mature T_2 seed of *B. carianta* transgenic line 14–8 expressing the *Lim Des 5* gene.

Table 3. Fatty acid composition of soybean seeds from non-transformed plants (*Non-transformed*) and plants transformed with the *Lim Des5* desaturase and *Lim FAE1* fatty acid elongase cDNAs (+ *Des5*/+ *FAE*).

Fatty acid	Non-transformed (n = 7)	+ *Des5*/+ *FAE* (n = 8)
	Weight % of total fatty acids	
16:0	11.2 ± 0.8	4.2 ± 0.2
16:1$^{\Delta 5}$	ND[a]	0.1 ± 0.1
18:0	3.7 ± 0.3	1.7 ± 0.1
18:1$^{\Delta 9}$	9.5 ± 1.8	12.4 ± 1.2
18:1$^{\Delta 11}$	1.7 ± 0.2	0.8 ± 0.1
18:2$^{\Delta 9,\Delta 12}$	61.6 ± 1.2	46.5 ± 1.7
18:3$^{\Delta 9,\Delta 12,\Delta 15}$	12.2 ± 1.9	8.7 ± 0.6
20:0	0.2 ± 0.1	1.5 ± 0.1
20:1Δ5	ND	10.6 ± 0.8
20:1$^{\Delta 11}$	0.1 ± 0.1	10.0 ± 1.0
20:1$^{\Delta 13}$	ND	0.5 ± 0.1
20:2$^{\Delta 11,\Delta 14}$	ND	2.1 ± 0.2
22:0	0.1 ± 0.1	0.1 ± 0.1
22:1$^{\Delta 5}$	ND	0.4 ± 0.1
22:1$^{\Delta 13}$	ND	0.3 ± 0.1
22:2$^{\Delta 5,\Delta 13}$	ND	0.2 ± 0.1
Total C_{20} + C_{22}	0.4	25.7

[a]ND, Not detected.
Measurements were obtained from the analysis of seven to eight single seeds from each line ± SD.

amounts of C_{20} and C_{22} fatty acids was also observed between TAG and the major phospholipids. The total content of C_{20} and C_{22} fatty acids in PC and PE was approximately 9% and 5%, respectively, but >25% in TAG.

Discussion

In transgenic *B. carinata*, the most prominent polyunsaturated fatty acid product with a Δ5 unsaturation was 22:2$^{\Delta 5,\ \Delta 13}$ at about 11% (Table 4). There was a concomitant reduction of erucic acid to about 32%, compared to non-transformed *B. carinata* wherein erucic acid is typically about 42%. That the 22:2$^{\Delta 5,\Delta 13}$ synthesis occurred primarily at the expense of 22:1$^{\Delta 13}$ strongly supports that 22:1$^{\Delta 13}$ is the preferred monounsaturated substrate for the Δ5 desaturase.

In contrast to the results from *B. carinata*, only trace amounts of 22:2$^{\Delta 5,13}$ were detected in soybean seeds that were co-transformed with the *Lim Des5* and *Lim FAE1* cDNAs linked to strong seed-specific promoters. The major fatty acid of the transgenic soybeans was instead 20:1$^{\Delta 5}$, which

Table 4. Fatty acid composition of triacylglycerol (TAG), phosphatidyl choline (PC), and phosphatidylethanolamine (PE) in soybean seeds co-expressing the *Lim Des5* and *Lim FAE1* cDNAs.

Fatty acid	TAG (n = 3)	PC (n = 3)	PE (n = 3)
Weight% of total fatty acids			
16:0	4.2 ± 0.2	8.9 ± 0.8	18.8 ± 1.2
18:0	2.0 ± 0.1	2.8 ± 0.4	3.1 ± 0.2
$18:1^{\Delta 9}$	14.5 ± 0.5	9.7 ± 2.6	8.6 ± 2.8
$18:2^{\Delta 9,\Delta 12}$	45.6 ± 0.8	62.8 ± 4.5	58.7 ± 4.4
$18:3^{\Delta 9,\Delta 12,\Delta 15}$	5.1 ± 0.9	6.1 ± 1.2	4.9 ± 0.7
20:0	2.4 ± 0.1	0.9 ± 0.3	1.2 ± 0.5
$20:1^{\Delta 5}$	10.8 ± 0.1	3.2 ± 0.5	1.5 ± 0.2
$20:1^{\Delta 11}$	10.4 ± 0.1	2.9 ± 0.3	1.4 ± 0.1
$20:2^{\Delta 11,\Delta 14}$	2.1 ± 0.3	1.4 ± 0.1	0.9 ± 0.1
Other[a]	≤ 2.5	≤ 1.1	≤ 0.6
Total $C_{20}+C_{22}$	27.0	8.5	5.1

Measurements are expressed as weight% of total fatty acids of each lipid class ± SD.
[a]Includes $16:1^{\Delta 5}$, $18:1^{\Delta 11}$, $20:1^{\Delta 13}$, 22:0, $22:1^{\Delta 5}$, $22:1^{\Delta 13}$, and $22:2^{\Delta 5,\ \Delta 13}$.

is also the primary fatty acid of *L. douglasii* seeds. Based-on the pathway previously described by Pollard and Stumpf (1980), this fatty acid is presumably synthesized by (1) elongation of 16:0-CoA and 18:0-CoA to 20:0-CoA, which is initiated by the activity of FAE1, and (2) subsequent Δ^5 desaturation of 20:0-CoA by the activity of Des5. Most strikingly, amounts of 16:0 and 18:0 were reduced by >2.5-fold in seeds from the transgenic line relative to those from non-transformed plants (Table 3). The reductions in 16:0 and 18:0 content in seeds of the transgenic soybean plants were, in fact, approximately 2-fold greater than those observed previously in soybean somatic embryos co-transformed with the *L. douglasii* cDNAs. The decrease in 16:0 and 18:0 levels is consistent with our previous demonstration that the *Lim FAE1* elongates saturated fatty acids in preference to monounsaturated fatty acids. Of note, amounts of $20:1^{\Delta 11}$ accumulated in the transgenic soybean seeds were only slightly less than those of $20:1^{\Delta 5}$ (Table 2). This result differs from those previously obtained using soybean somatic embryos (Cahoon et al. 2000). In our previous experiments with soybean somatic embryos, accumulated levels of $20:1^{\Delta 5}$ were 4- to 5-fold greater than those of $20:1^{\Delta 11}$. One possible explanation for the results observed in the transgenic soybean seeds is that as pool sizes of 16:0 and 18:0 are depleted by the activity of the *L. douglasii FAE1*, this enzyme increasingly uses $18:1^{\Delta 9}$ as an alternative substrate, resulting in the production of $20:1^{\Delta 11}$. Based on this, we speculate that, in contrast to *L. douglasii* seeds, the production of 16:0 and 18:0 in soybean seeds is limiting for high levels of flux into the 20:0 substrate for the *L. douglasii* Des5.

In general, C_{20} and C_{22} fatty acids, including the novel fatty acids, $20:1^{\Delta 5}$ and $22:2^{\Delta 5,\Delta 13}$ were not excluded from the major phospholipid classes. However, the relative amounts of $20:1^{\Delta 5}$ in soybean PC and of $22:2^{\Delta 5,\ \Delta 13}$ in *B. carinata* PC were about 4- and 10-fold lower than the respective proportions of these fatty acids found in TAG of these transgenic lines. It is interesting to note that PC from mature *B. carinata Des5* transgenic lines -contained significant amounts of erucic acid. In previous studies of seeds of *Brassicaceae, Limnanthaceae* and *Tropaeolaceae* which accumulate high proportions of VLCFAs in their TAGs, incorporation of radiolabeled VLCFA moieties have been detected in PC and other polar lipids in metabolic studies of developing embryos (Pollard and Stumpf 1980a,b; Fehling et al. 1990; Lohden and Frentzen 1992; Taylor et al. 1992). However, these proportions are typically greater than the level of VLCFAs found in PCs of developing embryos on a mass basis. Furthermore, VLCFAs are usually absent or found in only trace proportions in the PC fraction of mature seed. There is evidence that, in general, VLCFAs are typically excluded from polar glycerolipids such as PC, and it has been suggested that such partitioning ensures that proper membrane functions are not disrupted by

the aberrant structures induced by these 'unusual' acyl moieties (Stymne et al. 1990). Indeed, transgenic *Arabidopsis* plants transformed with a 35S-*FAE1* construct accumulated very high proportions (up to 30%) of VLCFAs (eicosenoic and erucic acids) in all major leaf membrane lipids. These transgenic lines displayed a dramatically altered morphology which included failure of flowering shoots to bolt, a modified spatial pattern of silique development, an altered floral phenotype among other effects (Millar et al. 1998). The basis for the ability of the PC fraction to tolerate the significant levels of VLCFAs found in the mature transgenic *B. carinata* or soybean seed (about 10% and 8%, respectively), is not known.

The *Lim Des5* substrate specificity for VLCFAs was almost exclusively restricted to 20:0 or 22: $1^{\Delta 13}$ and did not show a significant affinity for 20:1. Indeed, when the *Lim Des5* was expressed in *Arabidopsis thaliana*, there was no significant production of $20{:}2^{\Delta 5,\Delta 11}$ even though the potential substrate, $20{:}1^{\Delta 11}$ represented almost 20% of the total seed fatty acids (data not shown).

Limnanthes species are part of the USDA's New Crop initiative. However, *Limnanthes douglasii* oil content is low, typically in the range of 20–24% (http://www.ncaur.usda.gov/nc/ncdb/search.html-ssi), with $22{:}2^{\Delta 5,\Delta 13}$ proportions from 10 to 15%, giving this developing crop a $22{:}2^{\Delta 5,\Delta 13}$ content ranging from 2.0 to 3.6 mg/g DW. Thus, our *B. carinata Des5* transgenic prototype with about 30% oil and 10% 22:2 is already approaching the same range for total $22{:}2^{\Delta 5,\Delta 13}$ content (3.0 mg/g DW), making it a potential competitive source of this unique diene.

Brassica carinata is an attractive host for genetic manipulations to produce triacylglycerols high in strategic VLCFAs such as $22{:}2^{\Delta 5,\Delta 13}$ for several reasons: (1) it is naturally high in erucic acid (approximately 35–40%), the substrate for $22{:}2^{\Delta 5,\Delta 13}$; (2) *B. carinata* rarely out-crosses as compared with *B. napus* HEAR and canola varieties, and therefore does not require growth in isolation from these oilseed crops; and (3) *B. carinata* can be grown in more arid conditions and on more marginal agricultural land, which would allow specialty oilseed crop acreage to spread out along the more southern regions of Canada's Prairies, some areas of which are undergoing desertification.

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